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(54) **Thrombolytic composition and a process for production thereof.**

(57) The present invention is directed to a thrombolytic composition comprising tissue plasminogen activator and arginine or an acid addition salt thereof. Arginine or an acid addition salt thereof can increase the solubility of the tissue plasminogen activator, and thus the tissue plasminogen activator content in the thrombolytic composition can be enhanced.

EP 0 217 379 A2

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THROMBOLYTIC COMPOSITION AND A PROCESS FOR PRODUCTION THEREOF

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BACKGROUND OF THE INVENTION

The present invention relates to a thrombolytic composition and a process for production thereof.

Thrombolytic compositions have been hitherto developed from urokinase isolated and purified from urine or cultured mammalian kidney cells. Also, streptokinase extracted from ß-hemolytic streptococcus has been provided for practical use. However, urokinase has a poor affinity to thrombus so that a large dose of urokinase is required to achieve the desired therapeutic effects. Particularly with systemic administration, bleeding has been feared due to destruction of coagulation factors by large quantities of plasmin induced in the blood. Tissue plasminogen activator (hereafter referred to as t-PA) found in human or other animal tissue or tissue culture medium derived from human or other animal tissues or tumor cells has a higher affinity to thrombus and a higher thrombolytic activity as compared to urokinase. Therefore, t-PA has been expected to give desired therapeutic effects by administration in a lesser dose and as a new thrombolytic agent. In addition, it has recently been attempted to produce t-PA by genetic engineering techniques.

The present inventors have purified t-PA and investigated a process for producing a thrombolytic composition comprising t-PA as an effective component. However, it has been clarified that the solubility of t-PA decreases as the degree of purification of t-PA becomes higher, and such is a serious obstacle to medical preparations.

BNSDOCID: <EP 0217379A2>

SUMMARY OF THE INVENTION

The present invention is directed to a thrombolytic composition which includes arginine or an acid addition salt thereof to increase the solubility of t-PA, and a process for production thereof.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a graph showing the relationship between the solubility of t-PA and concentration of arginine.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present inventors have found that the solubility of t-PA is markedly increased by the use of a solvent system containing arginine or an acid addition salt thereof.

Arginine used in the present invention can be any of D-, L- and racemic-form and may further be an acid addition salt thereof, e.g., hydrochloride thereof (hereafter the term arginine includes all of the above, unless otherwise indicated). The amount of arginine necessary for enhancing the solubility of t-PA is approximately 1 mM to 500 mM, preferably 5 mM to 200 mM. Even when the amount exceeds 500 mM, the system is effective but an increase in the solubility of t-PA corresponding to the increase in the amount of arginine is not expected. Therefore, the amount of 500 mM or less is most practical. Further, it is more preferred to use neutral salts, especially sodium chloride in a concentration of 0.02 M to 2.0 M, preferably 0.1 to 1.0 M, in combination with arginine. It is preferred that the pH of a t-PA solution containing arginine or arginine and neutral salts such as sodium chloride, etc., be maintained in a range of 2 to 12,

more preferably 6 to 11, by adding buffer solution of, e.g., sodium phosphate.

The thrombolytic composition of the present invention comprises at least arginine and t-PA as the effective components. Other components, e.g., excipients, stabilizers, etc., conventionally used in medical preparations, for example albumin, mannitol, gelatin, sodium chloride, etc., may be optionally incorporated.

The thrombolytic composition of the present invention can be prepared by dissolving purified t-PA in a solvent containing arginine and then packing the sterilized solution in an ampule, a vial, etc. If necessary, freeze-drying can be performed. Instead of dissolving t-PA in a solvent containing arginine, t-PA and a determined amount of arginine may be weighed and dissolved in a suitable solvent or packed in a suitable container, if necessary, together with other components, to prepare the thrombolytic composition. The solvents used in the present invention include distilled water for injection, saline, 0.01 M to 0.1 M phosphate buffer, and other pharmaceutically acceptable solvents. As the medical preparation, an injection is generally advantageous, but as long as the thrombolytic activity of t-PA can be maintained, any medical preparation can be used. The dose of the thrombolytic composition according to the present invention is generally 20,000 to 50,000 IU/kg for an adult, but can be appropriately varied depending upon conditions.

As stated above, by using the solvent containing arginine, it is possible to prepare a t-PA solution of high concentration. Consequently, it is possible to pack large quantities of t-PA in a container of a small volume, e.g., a vial or an

BNSDOCID: <EP 0217379A2>

ampule, and thus a therapeutically effective thrombolytic composition can be produced. For example, as apparent from Table 1 described below, the solubility limit of t-PA in saline is around 15,000 IU/ml, so that it is difficult even to produce preparations of 100,000 IU/vial. However, when the solvent is saline containing arginine hydrochloride, it is easy to produce preparations of around 2,000,000-5,000,000 IU/vial. Accordingly, it is extremely useful in medical preparation of t-PA to incorporate arginine in the t-PA solution.

The present invention will be described in more detail with reference to experiments and examples, but the invention is not deemed to be limited to these examples. The following experiments and examples use only t-PA purified from culture medium of human melanoma cells and Chinese hamster ovary (hereafter referred to as CHO) cells to which t-PA gene was transferred by a genetic engineering technique, but t-PA may be any of t-PA derived from human or other animal tissue, t-PA purified from a cell culture derived from human or other animal tissue, t-PA obtained by genetic engineering techniques other than the aforesaid techniques, such as t-PA obtained from microorganisms such as t-PA transferred eucaryocyte, Escherichia coli, Bacillus subtilis, yeast, etc., to which t-PA gene is transferred.

The measurement of the activity of t-PA was performed by the fibrin plate method using 95% clottable fibrinogen (plasminogen content, ca. 50 casein unit/g clottable protein), using t-PA (approved by WHO) as a standard.

Preparation 1 of t-PA

About 60 liters of a crude t-PA culture medium produced from a human melanoma cell culture in accordance with the

RNSDOCID: <EP 0217379A2>

D. Collen et al method (The Journal of Biological Chemistry, 256(13), 7035-7041, 1981) were purified with reference to the D.C. Rijken et al method (Thromb Haemosta, 48(3), 294-296, 1982). Namely, after purifying by Zn-chelate chromatography (13.5 x 17.5 cm), the culture medium was further applied to Concanavalin A-Sepharose chromatography (5 x 30 cm). t-PA was eluted with a 2.0 M potassium thiocyanate solution containing 0.4 M α-D-methylmanoside from the Concanavalin A-Sepharose column. After concentrating the eluted t-PA with polyethylene glycol, gel filtration was performed through a column (7.5 x 90 cm) packed with Sephacryl S 200 (made by Pharmacia) equilibrated with a 0.01 M phosphate buffer (pH 7.5) containing 0.25 M arginine hydrochloride to give purified t-PA. 18,000,000 IU of purified t-PA showing a specific activity of 2.5×10^5 IU/mg was obtained by the said purification steps. The thus obtained t-PA was dialyzed to distilled water at 4°C overnight and then freeze-dried to provide the following experiments and examples.

Preparation 2 of t-PA

A t-PA gene was prepared according to the D. Pennica et al method (Nature, 301(20), 214-221, 1983). The aforesaid t-PA gene was transferred to CHO cells and then the CHO cells were cultured according to the method of R.J. Kaufman and P.A. Sharp (Journal of Molecular Biology, 159, 601-621, 1982). Thereafter, 40 liters of crude t-PA culture medium were obtained and said medium was purified in a manner similar to Preparation 1 to give 12,000,000 IU of purified t-PA showing a specific activity of 1.7×10^5 IU/mg.

BNSDOCID: <EP 0217379A2>

Experiment 1

Influence of the arginine concentration on the solubility of t-PA was examined. Five mg each of the purified t-PA obtained in Preparation 1 were weighed and dissolved in 0.5 ml of a solution of arginine having a concentration shown in Table 1. The activity of t-PA in the solution was measured to examine the solubility of t-PA. In case that t-PA was not completely dissolved but precipitates were formed, the activity in the supernatant was measured. The results are shown in Table 1 and Figure 1.

Table 1

Solvent System	Titer of Solution (supernatant)	Dissolved State
Saline	16,100 IU/ml	white turbid
Saline containing:		
1 mM L-arginine hydrochloride	57,000 IU/ml	white turbid
5 mM L-arginine hydrochloride	104,000 IU/ml	white turbid
25 mM L-arginine hydrochloride	372,000 IU/ml	white turbid
100 mM L-arginine hydrochloride	1,220,000 IU/ml	slightly white turbid
500 mM L-arginine hydrochloride	2,270,000 IU/ml	clear
1,000 mM L-arginine hydrochloride	2,200,000 IU/ml	clear

BNSDOCID: <EP 0217379A2>

Experiment 2

Influence of the kind of arginine on the solubility of t-PA was examined. One mg each of the t-PA purified in Preparations 1 and 2 was dissolved in 0.5 ml of a solution containing 25 mM of various arginines and the activity of t-PA in the solutions was measured in a manner similar to Experiment 1. The results are shown in Table 2.

Table 2

Solvent System	t-PA in Preparation 1 Titer of Solution (supernatant)	Dissolved State	t-PA in Preparation 2 Titer of Solution (supernatant)	Dissolved State
Distilled water containing:				
25 mM L-arginine	306,000 IU/ml	almost clear	246,000 IU/ml	almost clear
25 mM L-arginine hydrochloride	351,000 IU/ml	almost clear	254,000 IU/ml	almost clear
25 mM D-arginine hydrochloride	298,000 IU/ml	almost clear	225,000 IU/ml	almost clear
25 mM DL-arginine hydrochloride	314,000 IU/ml	almost clear	241,000 IU/ml	almost clear
25 mM D-arginine	245,000 IU/ml	almost clear	218,000 IU/ml	almost clear

Experiment 3

The solubility of t-PA in a solvent system containing L-arginine hydrochloride and sodium chloride was examined. In 0.5 ml of a solution containing L-arginine hydrochloride or combined L-arginine hydrochloride and sodium chloride, the pH of which had been adjusted, 1 mg or 5 mg of purified t-PA was

BNSDOCID: <EP 0217379A2>

dissolved. The activity of t-PA in the solutions was measured to examine the solubility of t-PA. The results are shown in Tables 3 and 4.

Table 3

Solvent System	Titer of Solution (supernatant)	Dissolved State
Distilled water containing 100 mM L-arginine hydrochloride, pH 7.0	1,220,100 IU/ml	slightly white turbid
0.01 M phosphate buffer containing 100 mM L-arginine hydrochloride and 0.14 M NaCl, pH 7.0	1,640,000 IU/ml	clear
0.01 M phosphate buffer containing 100 mM L-arginine hydrochloride and 0.30 M NaCl, pH 7.0	2,090,000 IU/ml	clear

Purified t-PA weighed: 5 mg

Amount of solvent: 0.5 ml

BNSDOCID: <EP 0217379A2>

Table 4

Solvent System	Titer of Solution (supernatant)	Dissolved State
Distilled water containing 25 mM L-arginine hydrochloride and 0.14 M NaCl, pH 2.0 (adjusted with HCl)	187,000 IU/ml	slightly white turbid
Distilled water containing 25 mM L-arginine hydrochloride and 0.14 M NaCl, pH 4.0 (adjusted with citric acid)	291,000 IU/ml	white turbid
0.05 M phosphate buffer containing 25 mM L-arginine hydrochloride and 0.14 M NaCl, pH 7.0	414,000 IU/ml	clear
Distilled water containing 25 mM L-arginine hydrochloride and 0.14 M NaCl, pH 9.0 (adjusted with NaOH)	396,000 IU/ml	clear
Distilled water containing 25 mM L-arginine hydrochloride and 0.14 M NaCl, pH 11.0 (adjusted with NaOH)	407,000 IU/ml	clear

Purified t-PA weighed: 1 mg

Amount of solvent: 0.5 ml

Experiment 4 General safety and pyrogen test

The medical preparations prepared in Example 1 and Example 2 below were dissolved in saline or distilled water for injection and each solution was given to mice and guinea pigs at a dose of 500,000 IU/kg. No abnormality was observed in any of

BNSDOCID: <EP 0217379A2>

the mice or guinea pigs. 200,000 IU/kg of the same preparations were administered to rabbits to perform a pyrogen test. All of the preparations were negative.

As is evident from the foregoing experiments, arginine is useful for enhancing the solubility of t-PA.

Example 1

t-PA	5,000,000 IU
L-arginine hydrochloride	21 mg
Sodium phosphate	173.9 mg
Purified gelatin	100 mg

Each component described above was dissolved in 10 ml of distilled water for injection. After aseptic filtration, 0.1 ml of each was packed in a vial and freeze-dried to prepare a thrombolytic composition.

Example 2

t-PA	5,000,000 IU
L-arginine hydrochloride	52.5 mg
Sodium phosphate	173.9 mg
Sodium chloride	64.3 mg
Human serum albumin	20 mg

Each component described above was weighed and a thrombolytic composition was prepared in a manner similar to Example 1.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

EPODOC: EP 0217379A2

CLAIMS:

1. A thrombolytic composition comprising tissue plasminogen activator and at least one member selected from the group consisting of arginine and an acid addition salt of arginine.

2. A thrombolytic composition as claimed in claim 1, wherein said arginine is selected from the group consisting of D-arginine, L-arginine, and racemic-form arginine.

3. A thrombolytic composition as claimed in claim 1, wherein said acid addition salt of arginine is arginine hydrochloride.

4. A thrombolytic composition as claimed in claim 1, wherein said member is contained in an amount of 1 mM to 500 mM.

5. A thrombolytic composition as claimed in claim 1, wherein said tissue plasminogen activator is one produced by extraction and purification from human or other animal tissue or from a cell culture medium derived from human or other animal tissue.

6. A thrombolytic composition as claimed in claim 1, wherein said tissue plasminogen activator is one produced by extraction and purification from a culture medium of human melanoma cells.

7. A thrombolytic composition as claimed in claim 1, wherein said tissue plasminogen activator is one produced by culturing Chinese hamster ovary cells to which a tissue plasminogen activator gene has been transferred.

BNSDOCID: <EP 0217379A2>

8. A thrombolytic composition as claimed in claim 1, wherein said tissue plasminogen activator is one produced by culturing a microorganism selected from the group consisting of eucaryocyte, Escherichia coli, Bacillus subtilis and yeast, to which tissue plasminogen activator gene has been transferred.

9. A process for producing a thrombolytic composition, comprising packing a tissue plasminogen activator in a container for medical preparation in the presence of arginine or an acid addition salt of arginine.

10. The use of tissue plasminogen activator and at least one member selected from the group consisting of aginine and an acid addition salt of aginine to prepare a pharmaceutical for the treatment of trhombosis.

BNSDOCID: <EP 0217379A2>

1. A process for preparing a thrombolytic composition characterized by mixing a tissue plasminogen activator and at least one member selected from the group consisting of arginine and an acid addition salt of arginine.

2. The process according to claim 1, wherein said arginine is selected from the group consisting of D-arginine, L-arginine, and racemic-form arginine.

3. The process according to claim 1, wherein said acid addition salt of arginine is arginine hydrochloride.

4. The process according to claim 1, wherein said member is contained in an amount of 1 mM to 500 mM.

5. The process according to claim 1, wherein said tissue plasminogen activator is one produced by extraction and purification from human or other animal tissue or from a cell culture medium derived from human or other animal tissue.

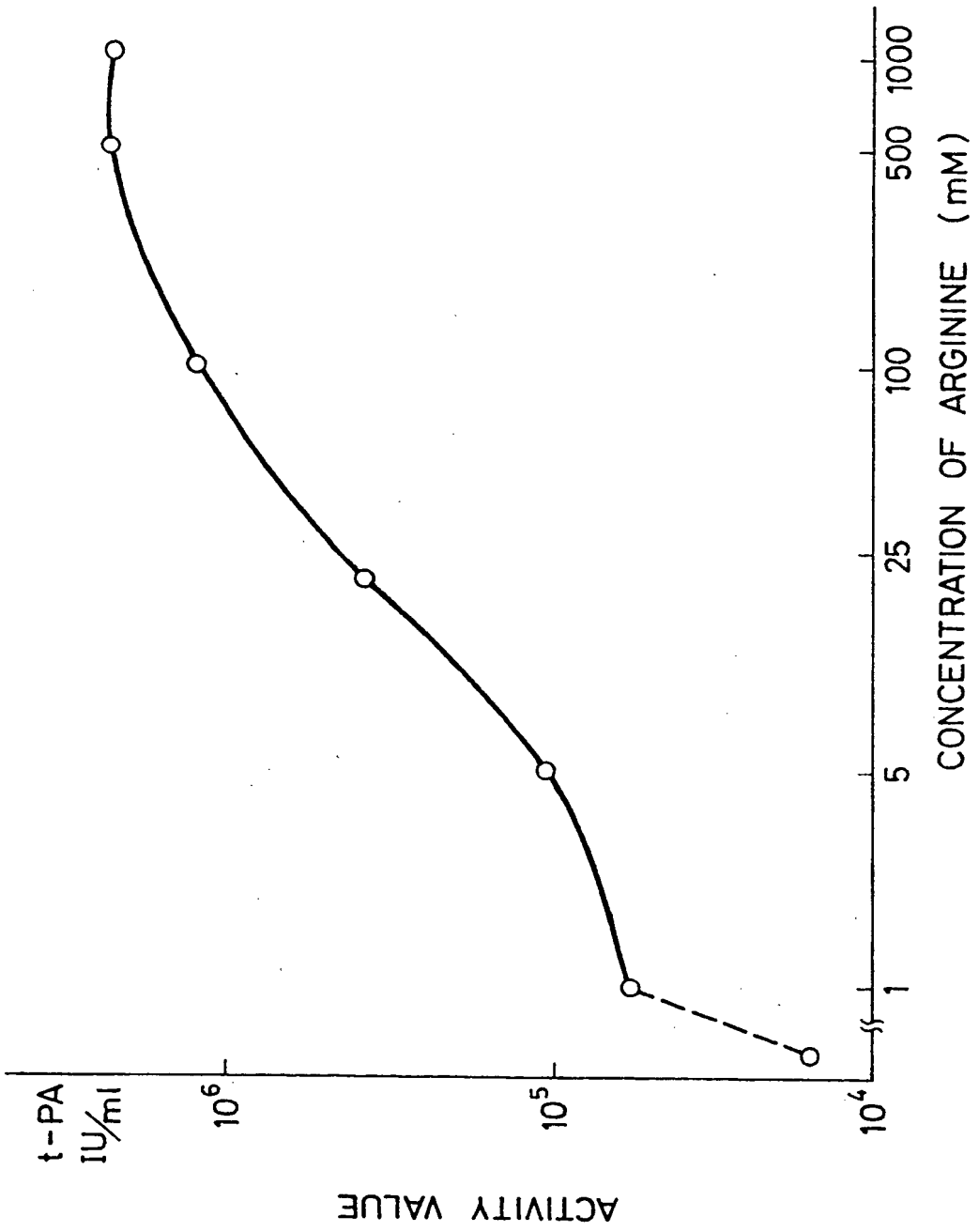
6. The process according to claim 1, wherein said tissue plasminogen activator is one produced by extraction and purification from a culture medium of human melanoma cells.

BNSDOCID: <EP 0217379A2>

7. The process according to claim 1, wherein said tissue plasminogen activator is one produced by culturing Chinese hamster ovary cells to which a tissue plasminogen activator gene has been transferred.

8. The process according to claim 1, wherein said tissue plasminogen activator is one produced by culturing a microorganism selected from the group consisting of eucaryocyte, Escherichia coli, Bacillus subtilis and yeast, to which tissue plasminogen activator gene has been transferred.

FIG. 1



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EP 0 217 379 A3

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0217379

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EUROPEAN SEARCH REPORT

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
E	EP-A-0 218 112 (EISAI CO.) * Page 3, lines 5-16; page 5, lines 13-18; page 9, table 1 *	1-10	A 61 K 37/54 // C 12 N 9/64 C 12 N 15/00
E	EP-A-0 228 862 (GENENTECH, INC.) * Page 3, lines 10-22; page 6, lines 13-29; pages 12-13, example 6 *	1-10	
A	GB-A- 985 498 (NOVO TERAPEUTISK LABORATORIUM) * Page 2, column 1, lines 20-29; page 3, table 1 *	1-10	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

A 61 K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	03-06-1988	FERNANDEZ Y BRANAS F.J.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

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BNSDOCID: <EP 0217379A3>